SPECIFIC INHIBITION OF REGENERATION IN CLYMENELLA TORQUATA[1]

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The notion that the extent to which an organ or tissue differentiates and grows may be limited by factors produced within the tissues themselves or by other tissues is relatively old. Hans Spemann first mentioned it in 1904, in discussing the results of his experiments on lens formation in *Triton*. E. I. Werber again stated the hypothesis in 1918, in discussing the phenomenon of lens regeneration in the salamander (p. 247): "According to our assumption it might perhaps be imagined that every tissue (or structure) elaborates during its development a substance (an 'antibody') which inhibits its growth beyond certain limits." H. W. Rand (1924) came to the same conclusion, apparently independently, in studying the regeneration of *Hydra* and *Planaria*. However, this hypothesis and its interesting implications became submerged in the wake of the exciting search for specific inductors which was stimulated by the brilliant experiments of Hans Spemann and his students.

C. M. Child, however (*e.g.*, 1929), maintained throughout the years that dominance and subordination are important forces acting in the development of the patterned individual. The hypothesis lay forgotten until 1952, when it was again restated by S. M. Rose and by Werner Braun, working independently on widely differing organisms. Rose worked on *Tubularia* and tadpoles, while Braun investigated population changes in bacteria. The next year, Gustafson (1953) arrived at the same hypothesis, also independently, while studying sea urchin development.

Since that time, much evidence has accumulated to support the dominance-inhibition theory of regeneration and differentiation, and the reader may consult Rose (1957) for a comprehensive review.

The present investigations have been undertaken in an effort to extend the validity of this theory to the annelids, the organism chosen in this case being the 22-segment polychaete, *Clymenella torquata*. This worm has been chosen for three reasons: (1) Sayles (1942) showed that the anterior and posterior ends of the worm possess differing capacities to respond to a regenerative stimulus; (2) *Clymenella* is an intertidal form, living in sandy mud in the Woods Hole region. Not only is it plentiful, but being an intertidal form, it possesses great capacity to withstand wide variations in temperature, stagnant water, and low oxygen tensions. It is thus an ideal laboratory animal. (3) In addition, *Clymenella* regenerates rather quickly at 15° C., results being obtainable in five to ten days.

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Methods

General

The worms for these experiments were collected from Barnstable Harbor, Barnstable, Mass., from the entrance to Squeteague Harbor, North Falmouth, Mass., and from the Northwest Gutter entrance to Hadley's Harbor, Naushon Island, Mass., by the M.B.L. Supply Department and by the investigator. Only adult worms, measuring 7–9 cm. in length, were used. For tests of posterior specificity, single segments (17 through 19) were isolated and cultured. For tests of anterior specificity, two-segment pieces (4 and 5, and 6 and 7) were obtained. All test and control pieces were cultured at 15° C. in a solution of 10 mg.% Chloromycetin and 50 units/cc. Mycostatin (E. R. Squibb) in filtered, pasteurized sea water (this solution is hereafter referred to as "treated water"). For the experiments, extracts were prepared by homogenizing the selected tissue in a glass homogenizer, using ice cold treated water as a solvent. These homogenates were then centrifuged at 5000 *g* for ten minutes, to remove large cellular debris, and added immediately to the isolated worm segments in the culture solution. Unless otherwise noted, the varying ratios of tissue to solvent appearing below were devised in an attempt to keep the tissue weight per ml. of solvent as nearly constant as possible for all tissues tested.

Series A. Test for posterior specificity

In this series of experiments, 161 posterior segments were isolated and divided into three groups. Forty-four segments were placed in individual containers (the depressions of an Hutzler ice ball tray) containing 4 ml. of treated water and 1 ml. of an extract prepared in the ratio of 1 prostomial piece (the prostomium was cut off at the level of the mouth, or just anterior to it) to 3 ml. of treated water. Sixty-four isolates were cultured in the same manner in depressions containing 4 ml. of treated water and 1 ml. of an extract of anal collars prepared in the ratio of one collar per ml. treated water (the anal collar of *Clymenella* contains almost exactly one-third as much tissue as the prostomium). The remaining 53 isolates were cultured as controls in 5 ml. of treated water alone. The segments were examined after 10 days.

Series B. Test for dorsal-ventral specificity within the anal collar

Here, 244 posterior segments were isolated. Ninety-eight were cultured in 4 ml. of treated water plus 1 ml. of an extract made from the dorsal halves of anal collars in a concentration of one dorsal half per ml. Ninety-eight others were treated in exactly the same manner, except that the test extract was made from the ventral halves of anal collars in a ratio of one ventral half per ml. treated water. The remaining 48 isolates were placed in 4.5 ml. of treated water plus 0.5 ml. of an extract of 2.4 ventral halves/ml. treated sea water. This slight increase in final extract concentration was obtained to check the possibility that the results of the immediately preceding series of tests with ventral half extracts represented a borderline concentration phenomenon. Examination of the test segments was made after 10 days.

Series C. Test for anterior-posterior gradient of tail-inhibiting substance

(1) In the first group of tests, 28 fifteenth and 28 seventeenth segments, placed in individual trays containing 4 ml. of treated water, received 1-ml. aliquots of an extract of 12 sixteenth segments per 32 ml. of treated water. In this way, the fifteenth segments were treated with material normally posterior to them, and the seventeenth, with material normally anterior to them. (2) In the second group, in order to provide clearer results, material from more distant regions was used, and in higher concentration. Fourteen sixteenth segments, cultured in 4 ml. of treated water, received 1-ml. aliquots of an extract of 14 fourteenth segments in 15 ml. of treated water. Fourteen fifteenth segments, cultured in 4 ml. of treated water, received 1-ml. aliquots of an extract of 14 seventeenth segments in 15 ml. of treated water. The test segments in groups 1 and 2 were cultured at a slightly elevated temperature (19° C.), and the results were therefore tabulated at the end of one week, rather than at the end of ten days.

Series D. Test for anterior specificity

Two hundred seventy two-segment anterior pieces were isolated for these experiments and divided into three groups. Ninety, placed in 4 ml. of treated water, received 1-ml. aliquots of an extract of prostomial pieces (obtained as in Series A) in a concentration of one piece per 3 ml. of treated water. Ninety more were cultured in 4 ml. of treated water plus 1 ml. of an extract prepared by grinding one anal collar per ml. of treated water. The remaining 90 isolates, serving as controls, were allowed to regenerate in 5 ml. of treated water alone. All segments were examined after ten days (cultured at 15° C.).

Series E. Tests for the electrophoretic mobility of the inhibitors

A lucite tray, with inside dimensions of 17.5 cm. × 8.5 cm. × 5 mm., was filled with 2% agar in sea water and prepared as in Figure 1. Three 7.5 cm × 5 mm. × 5 mm. transverse wells (H.W. in Figure 1) were cut into the agar to hold the homogenates to be tested. Four rows of 14 longitudinal wells 9 mm. × 2 mm. × 2 mm. (S.W. in Figure 1) were then cut into the agar 2 mm. away from the transverse wells. Into these wells were placed the test segments. Isolated posterior segments were placed so that their posterior cut surfaces always faced the nearest transverse well. Anterior isolates were arranged with their anterior cut surfaces facing the transverse wells. (This arrangement allowed the regenerating surface to be in the closest possible proximity to the homogenate.) With the segments in place, the longitudinal wells were filled with treated water and then covered with strips of wet knit cotton cloth to retard evaporation and drying of the test segments. The transverse wells were then filled with the appropriate homogenate or solution and covered with strips of polyethylene film. Subsequently, strips of Whatman no. 1 filter paper were moistened with sea water and placed at the ends of the agar tray, with their free ends hanging down. This whole apparatus was then lowered into place in an electrophoresis chamber surrounded with ice (to maintain a temperature of 15°–17° C. for the segments under treatment). The buffer wells were filled with cold sea water. A current

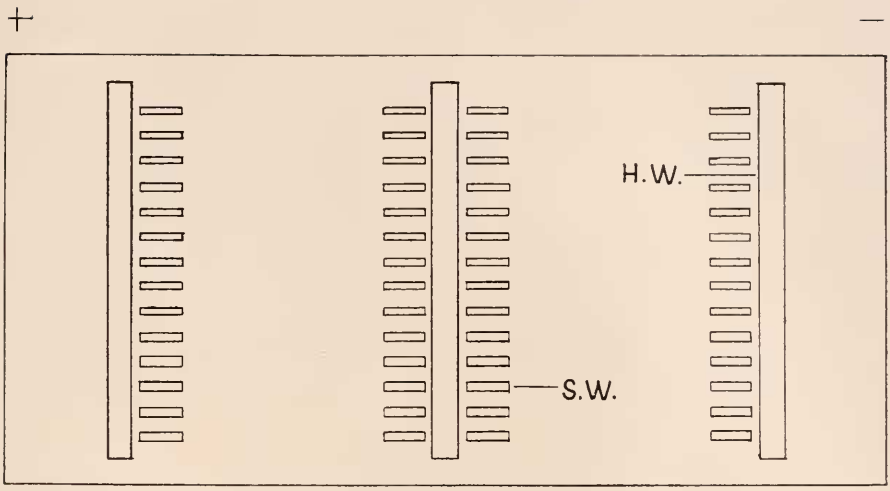

FIGURE 1. Electrophoresis tray; H.W., homogenate well; S.W., test segment well.

of 20 milliamperes at 15 volts was then applied with a VoKam constant current/constant voltage D. C. power supply (COLAB Instrument Co.) for four hours. After the four-hour treatment, the isolated segments were removed to individual trays containing 5 ml. of treated water. The segments were inspected after ten days, and the results were recorded. The experimental arrangements were as follows: (1) One hundred seventy-two posterior segments were divided into three groups. Fifty-six were placed in the apparatus as described above, and the homogenate wells were filled with an extract of 40 anal collars in 4 ml. of treated water (the higher concentrations used in this and the following experiments were selected in order to compensate for any loss of inhibitor during electrophoretic migration, and to ensure a steady supply of it to the test segment for the whole four hours of electrophoresis). Thus, 28 of these 56 segments were left with their posterior surfaces facing the positive pole, and 28 were left with posterior surfaces facing the negative pole. Fifty-six more segments acted as one control, being treated exactly as above, except that the homogenate wells were filled only with the treated water. The final 60 isolates were also treated exactly as above, except that the homogenate consisted of 20 prostomial pieces in 4 ml. of treated water. This last group served as a control against any possible cross-reactivity of the inhibitors. (2) One hundred seventy-two anterior two-segment isolates were also divided into three groups as above and treated with the same series of extracts and solutions in exactly the same concentrations and in exactly the same fashion as the posterior segments except that, as previously noted, the anterior cut surfaces faced the transverse homogenate wells.

RESULTS

Series A

The results of the tests for posterior specificity may be seen in Table I and Figure 2. Of the 53 control segments cultured in plain treated water, 8 had to be

discarded due to improper wound closure. (These discards are listed in Table I and in subsequent tables as "out.") In general, approximately 15% of the isolated posterior segments showed this defect. It was a result of the closing over of the posterior end of the gut tube. There followed the protrusion of a large, fluid-filled sac which pressed upon the surrounding tissue and effectively prevented regeneration. Of the remaining 45 controls, 44 produced perfect anal collars in ten days (Fig. 2A). Illustration (B) of Figure 2 shows a typical segment treated with prostomial extract. Of the 40 posterior segments so treated which remained healthy, 36 produced perfect tails, fully as large as those of the controls. The segments treated with anal collar extract, however, presented an entirely different picture, typified by drawing (C) of Figure 2. Not a single one of the segments which closed its wound properly regenerated a tail. The wound merely closed over, and the process stopped at that point. A few segments were

TABLE I

Posterior specificity

Treatment	No. cases	No. out	No. regenerates	% Regenerates
Control	53	8	44/45	97.7
Prostomial extract	44	4	36/40	90.0
Anal collar extract	64	10	0/54	0.0

allowed to progress for a month, and at the end of that time, the majority showed signs of recovery and regeneration, but within the limits of the experiment, no recovery was evident.

Series B

The results of this series of experiments are presented in Table II and Figure 3. The results, in this case, were somewhat less striking than in the previous series. Of the 98 posterior segments treated with dorsal collar extracts, 9 closed wounds improperly and were discarded. Twenty-nine of the remaining 89 pieces showed either no regeneration at all, or only slight accumulations of new tissue within the limits of the experiment. The remaining 60 pieces, however, when compared with a control segment ('A' of Figure 3) from the previous series, presented interesting results. As may be seen from part (B) of Figure 3, the dorsal segment of the regenerating anal collar was much reduced and lacked cirri. The situation which prevailed with the posterior segments receiving ventral collar extracts was much the same in nature as that above, except that the incidence of homologous inhibition was much lower, and the incidence of total inhibition much higher. Of the 98 segments treated with the 1:1 ventral collar extract, 13 were discarded. Forty showed homologous inhibition of the ventral half of the anal collar ('C' of Figure 3). Forty-five, however, were completely inhibited. These data suggested that perhaps the difference between the specific and non-specific total inhibitions might be a matter of concentration. Therefore, the second group of ventral collar extract tests was run with slightly higher final concentration (1.2:1). Of the 48 segments so treated, 7 were discarded, only

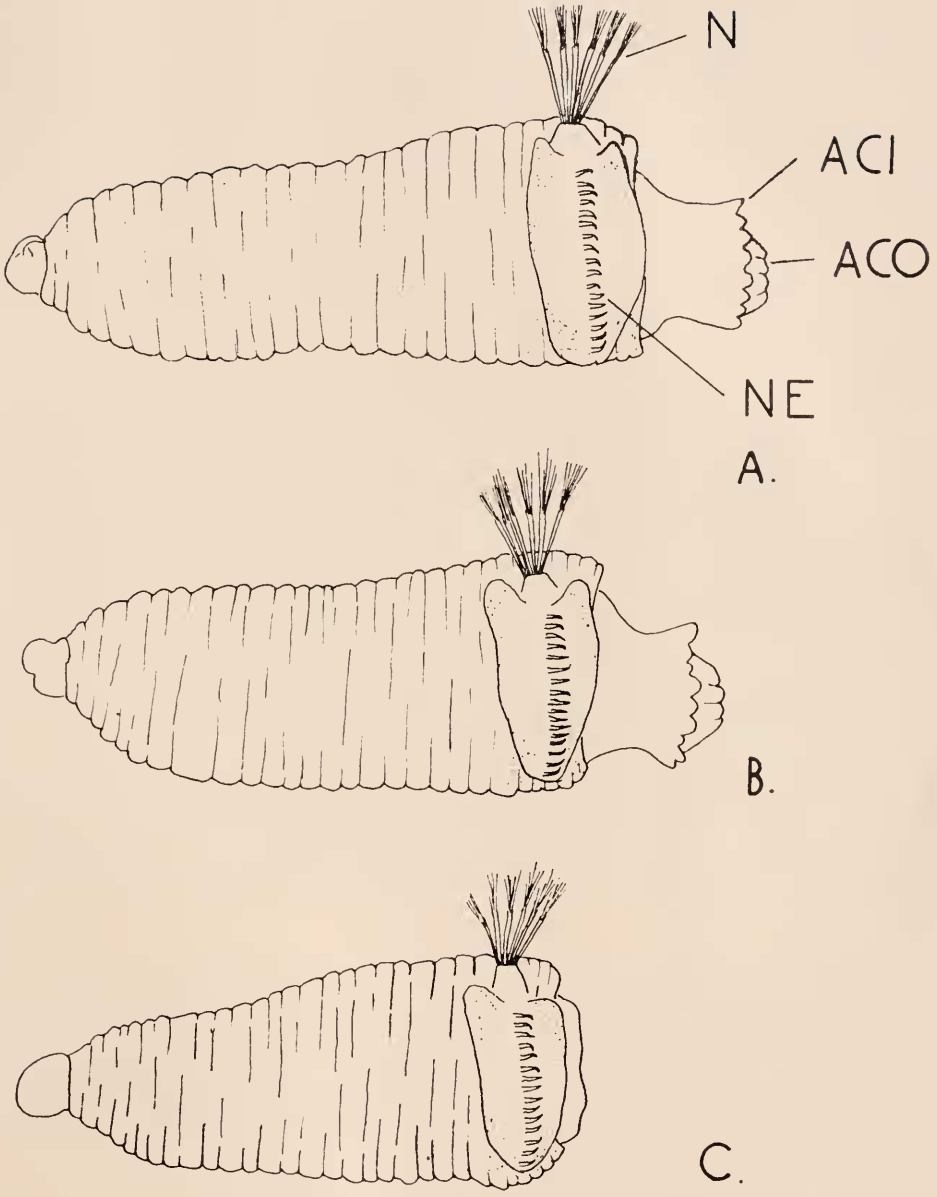

FIGURE 2. A. Posterior segment treated with culture water only. N, notosetae; NE, neurosetae; ACI, anal collar with cirri; ACO, anal cone (pygidium). B. Posterior segment treated with prostomial extract. C. Posterior segment treated with anal collar extract.

TABLE II

Dorsal-ventral specificity

Treatment	No. cases	No. out	No. totally inhibited	No. dorsally inhibited	No. ventrally inhibited
Dorsal collar extract	98	9	29	60	0
Ventral collar extract 1:1	98	13	45	0	40
Ventral collar extract 1.2:1	48	7	28	0	13

13 were homologously inhibited with reduced ventral segment, and the rest were totally inhibited.

Series C

Of the original 28 posterior segments treated with materials from the segment immediately posterior to them, 22 closed their wounds properly. Of these, only four proceeded to initiate regeneration, and this was so slow and late that within the seven-day limit of the experiment, no indications of normal anal collars and cirri appeared. The 28 pieces treated with extract of the immediately preceding segment presented a curious phenomenon. Fifteen of these pieces began to develop a peculiar blistering of the epidermis at the anterior end after two days. This condition progressed posteriorly and inwardly during the following five days, arriving finally at a point where the entire test segment appeared to be a mere sac of dissociated cells. These pieces did not regenerate. However, the remaining 13 test segments, though they began to show signs of the same affliction, proceeded to regenerate anal collars normally.

The 14 test pieces (segment 15) treated with extracts of segment 17 were inhibited without exception. One piece was discarded, and of the remaining 13, none developed new tissue within the seven-day period. One of the 14 sixteenth segments treated with segment 14 extracts was discarded. Five developed the blistering reaction noted above, but the eight which were left regenerated normally.

Series D

The 90 control segments of this series regenerated uniformly, 80 outgrowths, averaging 0.8 mm. in length, appearing, with only 10 failures. In this case, the failures were due to the fact that the isolating cuts were not precisely in the intersegmental plane. Under such conditions, regeneration failed. The appearance of a control segment may be seen in Figure 4A. Figure 4B and Table III show that

TABLE III

Anterior specificity

Treatment	No. cases	No. regenerates	No. failures
Control	90	80	10
Prostomial extract	90	15 (delayed)	75
Anal collar extract	90	76	14

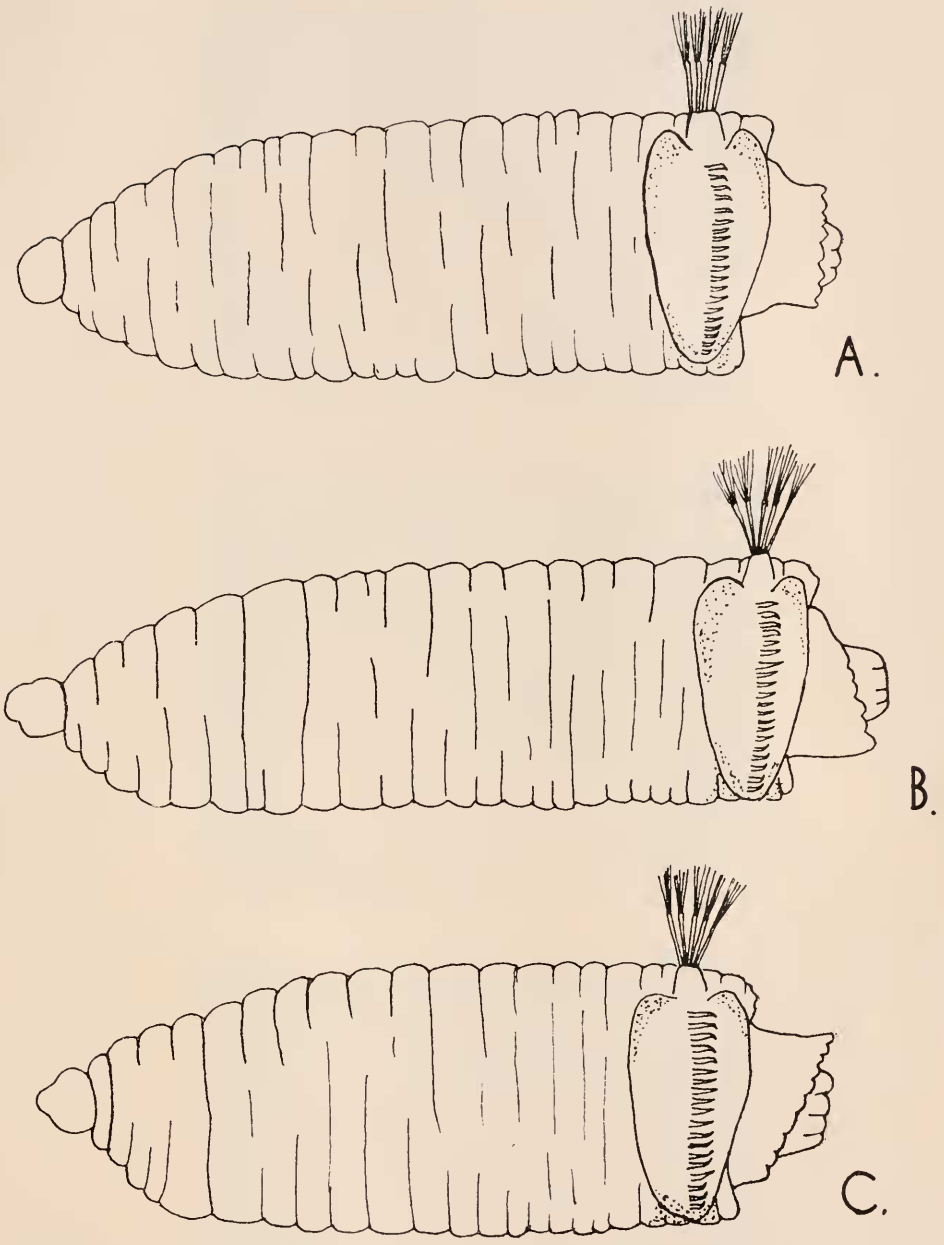

FIGURE 3. A. Posterior segment treated with culture water only. B. Posterior segment treated with dorsal collar extract. C. Posterior segment treated with ventral collar extract.

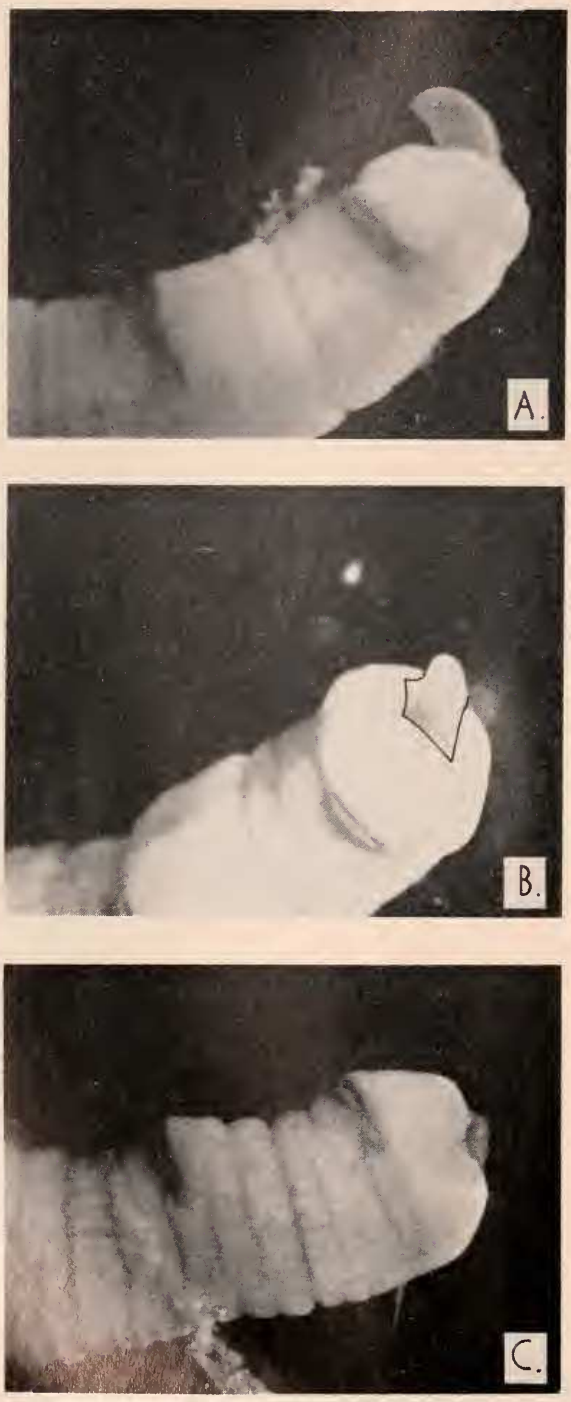

FIGURE 4. A. Anterior segments treated with culture water only. B. Anterior segments treated with anal collar extract. C. Anterior segments treated with prostomial extract.

the 90 pieces treated with tail extract regenerated almost as well as the controls. Seventy-six regenerated and 14 failed, presumably because of improper cutting. The 90 test pieces treated with prostomial extract fared very poorly. Only 15 of the 90 produced regenerates, and these were very small, none of them exceeding 0.2 mm. in length. Seventy-five failed to regenerate at all, though 10 to 15 of the failures were no doubt the result of improper cuts. Figure 4C shows one of these failures.

Series E

The results of the tests for electrophoretic mobility of the inhibitors may be seen from Table IV and Figures 5 and 6. Of the 172 posterior segments placed in the agar trays, only the 28 with the posterior cut surface facing the positive pole and a well filled with anal collar extract showed any evidence of specific inhibition. The other 144 were essentially unimpaired by the procedures and regenerated normally. Of course there were some failures due to improper wound

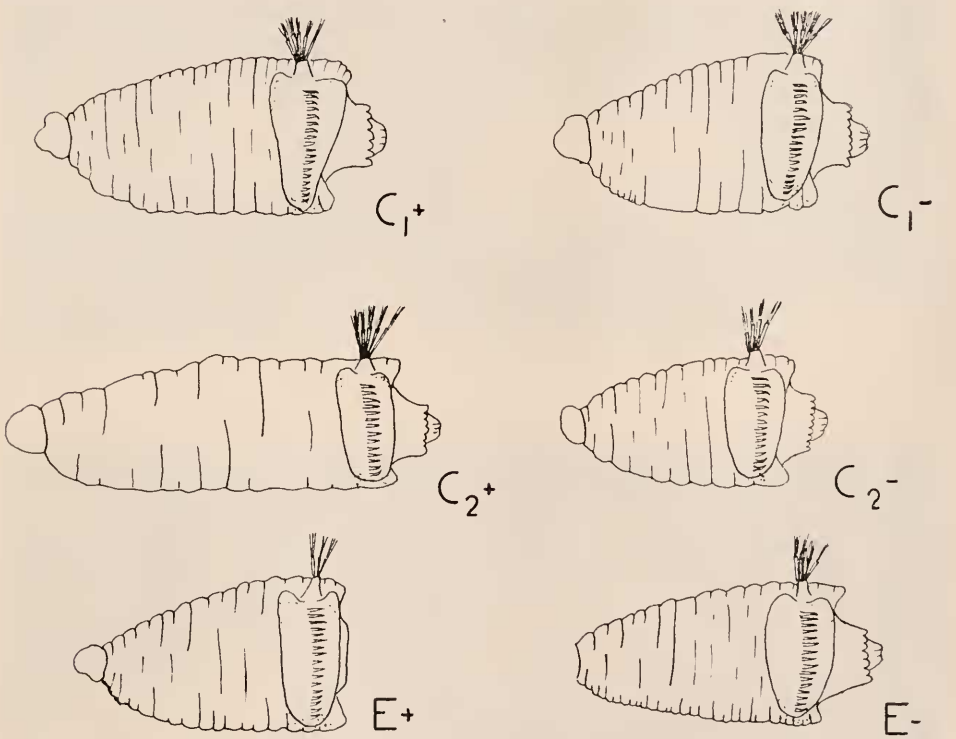

FIGURE 5. C_1+, posterior segment, posterior cut surface facing positive pole, treated water in homogenate well. C_1-, posterior segment, posterior cut surface facing negative pole, treated water in homogenate well. C_2+, posterior segment, posterior cut surface facing positive pole, prostomial extract in homogenate well. C_2-, posterior segment, posterior cut surface facing negative pole, prostomial extract in homogenate well. E+, posterior segment, posterior cut surface facing positive pole, anal collar extract in homogenate well. E−, posterior segment, posterior cut surface facing negative pole, anal collar extract in homogenate well.

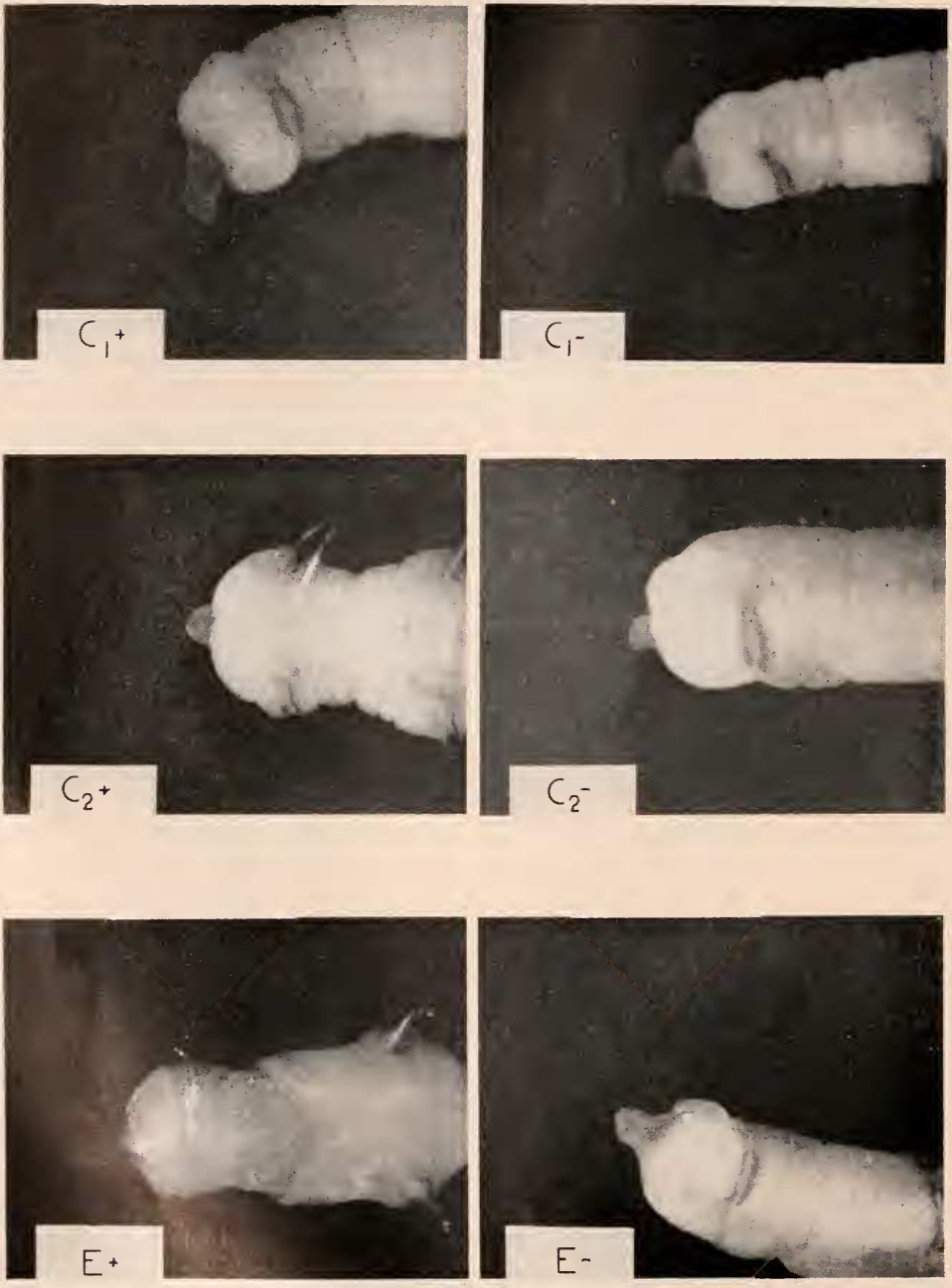

FIGURE 6. C_1+, anterior segments, anterior cut surface facing positive pole, treated water in homogenate well. C_1-, anterior segments, anterior cut surface facing negative pole, treated water in homogenate well. C_2+, anterior segments, anterior cut surface facing positive pole, anal collar extract in homogenate well. C_2-, anterior segments, anterior cut surface facing

TABLE IV

Electrophoretic mobilities

Segment	No. cases	Cut surface facing +	Cut surface facing −	Extract	No. out	No. regenerates	No. failures
Posterior	28	posterior	anterior	water	1	26	1
Posterior	28	anterior	posterior	water	1	27	0
Posterior	30	posterior	anterior	prostomial	3	24	3
Posterior	30	anterior	posterior	prostomial	2	26	2
Posterior	28	posterior	anterior	an. collar	0	2	26
Posterior	28	anterior	posterior	an. collar	1	25	2
Anterior	28	anterior	posterior	water	1	24	3
Anterior	28	posterior	anterior	water	3	22	3
Anterior	30	anterior	posterior	an. collar	0	27	3
Anterior	30	posterior	anterior	an. collar	2	27	1
Anterior	28	anterior	posterior	prostomial	0	0	28
Anterior	28	posterior	anterior	prostomial	1	26	1

closure. Similar results were obtained with the anterior test pieces. The 28 with anterior cut surfaces facing the positive pole and a well filled with prostomial extract showed total inhibition. The other 144 were unaffected (Fig. 6E).

DISCUSSION

The results of these investigations are relatively clear, and may be readily interpreted, except for one or two points. The results of the tests for anterior and posterior specificity may be interpreted as demonstrating the presence of two distinct moieties. One, produced in the anal collar, can specifically inhibit its regeneration, and presumably acts in a similar capacity to limit its size in normal development. A similar specific moiety may be postulated as arising in the prostomium during development, to check its growth. When extracted, this substance is also capable of checking head regeneration. Marie Tucker (1959) has demonstrated the presence of a similar pair of extractable head- and tail-inhibitors which are specific for their homologous structures in the nemertean, *Lineus vegetus*. Lender (1956) describes an inhibitor which is specific for the brain of *Dugesia lugubris*. He points out that tail extracts have no effect on the regeneration of this structure. The data gathered in these investigations almost certainly represent evidence of the same sort of phenomenon.

The results of the tests for dorsal-ventral specificity within the anal collar strongly suggest that the tail-inhibitor may be divided into at least two distinct inhibitors, one of which limits dorsal development, and one of which affects the ventral half of the collar. However, the high incidence of total inhibition with dorsal, and especially with ventral, extracts casts some doubts. It may perhaps be true that the dorsally and ventrally evolved inhibitors are quite specific in their actions. If this be the case, then one must postulate the presence of a third and

negative pole, anal collar extract in homogenate well. E+, anterior segments, anterior cut surface facing positive pole, prostomial extract in homogenate well. E−, anterior segments, anterior cut surface facing negative pole, prostomial extract in homogenate well.

more general inhibitor present in slightly lower concentration, to explain the increase in total inhibition with a slight increase in extract concentration. Perhaps a more likely explanation of the observed facts would assume that the dorsal and ventral inhibitors possess, in addition to their specific action, the ability to cross-react with their opposite halves if present in sufficiently high concentration. In any event, this dichotomy of possibilities exists and awaits further elucidation.

The results of the tests for an anterior-posterior gradient of tail-inhibiting substance provide evidence of two phenomena. Material from posterior segments, when placed in contact with more anterior segments, prevents them from regenerating a tail. Material from a level normally more anterior than the test segment does not inhibit tail regeneration. These facts demonstrate nearly conclusively the presence of a gradient of tail-inhibitory activity with its zenith at the posterior end of the worm. There is apparently also a gradient in the opposite direction. The large number of segments showing the blistering-disaggregation reaction when treated with extracts of a more anterior region, but not when treated with more posterior material, is a very interesting observation. One is tempted to postulate an anterior-to-posterior gradient of some kind of somatic tissue factor—perhaps the sort of factor which may determine "headness" and "tailness" in the worm, and/or which, by virtue of some sense of quantitative distribution, allows the worm to maintain its almost universally constant number of 22 segments. However, this obviously remains to be proved.

The results of the electrophoretic mobility tests clearly indicate that at sea water pH (*ca.* 8.1) both the head- and tail-inhibitors are positively charged. They move through the agar toward the negative pole and apparently enter the exposed surfaces of the test pieces. The electrical current itself obviously has no effect, nor is there any cross-reaction of the inhibitors. There is, of course, no valid reason for concluding that this positive charge is present within the worm's tissues, the pH there being unknown. Nor is it known that the two inhibitors possess the same degree of positivity. The possibility is apparent, however, that the inhibitors may be electrophoretically mobile within the worm. It would be very interesting to know what, if any, natural bioelectric polarities exist within this species, since electrical fields have been shown to be capable of controlling the polarity of growth in *Fucus* (Lund, 1923), *Obelia* (Lund, 1925), *Dugesia* (Marsh and Beams, 1952), and *Perophora* (Smith, unpublished data). Bioelectric fields have also been implicated in the control of amphibian limb regeneration (Becker, 1961). If one could correlate the posterior-anterior gradients of inhibitory activity with naturally occurring bioelectric fields, it would suggest that pattern and growth specificities might be due to the specific mobilization and concentration of substances within such fields.

Summary

1. Evidence is presented suggesting the presence of specific head- and tail-inhibitors, and of even more specific dorsal-ventral intra-organ tail-inhibitors.

2. The tail-inhibitor is shown to be distributed along a posterior-to-anterior gradient.

3. Another substance, a "somatic factor," is postulated as existing along an anterior-to-posterior gradient.

4. The electrophoretic mobilities of the head- and tail-inhibitors at sea water pH are demonstrated.

5. The possibility of bioelectric control of the movements and concentrations of growth- and pattern-specific substances is briefly considered.

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